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The human coronary vasodilatory response to acute mental stress is mediated by neuronal nitric oxide synthase.

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Running head: nNOS and coronary flow during mental stress

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ABSTRACT

Introduction. Mental stress-induced ischemia approximately doubles the risk of cardiac events in patients with coronary artery disease, yet the mechanisms underlying changes in coronary blood flow in response to mental stress are poorly characterized. Neuronal nitric oxide synthase (nNOS) regulates basal coronary blood flow in healthy humans and mediates mental stress-induced vasodilation in the forearm. However, its possible role in mental-stress-induced increases in coronary blood flow is unknown. *Methods.* We studied eleven patients (6 men, mean age 58 ± 14 years) undergoing elective diagnostic cardiac catheterization, and assessed the vasodilator response to mental stress elicited by the Stroop color-word test. Intra-coronary substance P (20 pmol/min) and isosorbide dinitrate (1 mg) were used to assess endothelium-dependent and -independent vasodilation respectively. Coronary blood flow was estimated using intra-coronary Doppler recordings and quantitative coronary angiography to measure coronary artery diameter. *Results.* Mental stress increased coronary flow by $34 \pm 7.0\%$ over the preceding baseline during saline infusion ($p < 0.01$), and this was reduced to $26 \pm 7.0\%$ in the presence of the selective nNOS inhibitor S-methyl L-thiocitrulline (SMTC, $0.625 \mu\text{mol/min}$; $p < 0.001$). Mental stress increased coronary artery diameter by $6.9 \pm 3.7\%$ ($p = 0.02$), and by $0.5 \pm 2.8\%$ ($p = 0.51$) in the presence of SMTC. The response to substance P did not predict the response to mental stress ($r^2 = -0.22$, $p = 0.83$). *Conclusion.* nNOS mediates the human coronary vasodilator response to mental stress, predominantly through actions at the level of coronary resistance vessels.

Keywords: mental stress, coronary, endothelial function, nitric oxide, human

New and Noteworthy: Acute mental stress induces vasodilation of the coronary microvasculature. Here, we show that this response involves neuronal nitric oxide synthase (nNOS) in the human coronary circulation.

INTRODUCTION

Acute mental stress can impact on cardiovascular morbidity and mortality, triggering myocardial infarction, ventricular arrhythmia and left ventricular failure (11). Analogous to exercise-induced stress, mental stress can exert detrimental effects due to sub-optimal coupling between myocardial oxygen demand and blood flow (31). The physiological cardiovascular response to mental stress mirrors that to other sympathetic stimuli and includes a catecholamine-driven increase in heart rate, blood pressure and cardiac contractility (17). Coronary blood flow increases in parallel to the higher myocardial oxygen demand, mediated mainly by reduction in coronary vascular resistance. Factors identified to mediate coronary vasodilator reserve include K_{Ca} channels (27), adenosine and nitric oxide (NO) (45). The vasomotor response to mental stress can be attenuated or even reversed in the presence of coronary artery disease; a condition that features endothelial dysfunction and reduced NO availability (9, 41, 43).

Until relatively recently, it was generally assumed that the NO responsible for mediating local increases in blood flow was generated by endothelial NO synthase (eNOS) expressed in endothelial cells (10, 16). However, studies using intra-arterial infusion of a selective neuronal NOS (nNOS) inhibitor show that local nNOS-derived NO is a major contributor to the basal regulation of microvascular tone and blood flow in the human forearm and coronary circulations (32, 33). It was also found that local nNOS is involved in mental stress-induced forearm vasodilatation in healthy humans. In this study, we have investigated the role of nNOS in the changes in coronary blood flow during acute mental stress in humans.

METHODS

The study was conducted in accordance with the Declaration of Helsinki and received ethical approval from the local Research and Ethics Committee. All participants provided written informed consent. Eleven patients (6 men, mean age 58 ± 14 years) undergoing elective diagnostic coronary angiography at King's College Hospital, London were included in the

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study. Patients had to have at least one angiographically unobstructed coronary artery as the study vessel. Those with a history of coronary disease, myocardial infarction, left ventricular impairment, dysrhythmia or renal impairment were excluded. Participants abstained from food for at least 6 hours prior to cardiac catheterization, and from all medication (except aspirin) on the day of the procedure.

Standard diagnostic coronary angiography was performed in a quiet, temperature-controlled cardiac catheterization laboratory with digital cineangiography. After completion of the diagnostic procedure, a 6F guide catheter was positioned in the study artery and a 0.014-inch intra-coronary Doppler wire (FloWire, Volcano Therapeutics, Rancho Cordova, CA) was advanced into a proximal segment that was free from side branches. The Doppler wire was interfaced with a real-time spectral analysis system (ComboMap Pressure and Flow system; Volcano Therapeutics) to record the average peak velocity (APV) of blood flow. APV recordings were taken prior to coronary angiography at each stage of the protocol, which was performed using non-ionic contrast medium (Omnipaque, GE Healthcare, Inc) and without altering the angle of projection during the study. Offline analysis was performed using an automated quantitative coronary edge detection system (Philips) to measure changes in epicardial coronary artery diameter in a 2.5 to 5mm length segment of vessel, ~2.5mm distal to the tip of the Doppler wire. Coronary flow was derived from the APV and arterial diameter (12).

All drugs were infused via the guide catheter into the study artery at a rate of 2ml/min. The selective nNOS inhibitor S-methyl L-thiocitrulline (SMTC) was obtained from Merck Millipore (Massachusetts, USA) and was prepared to Good Medical Practice standards for human use in a nationally accredited pharmaceutical manufacturing facility. Substance P was obtained from Bachem (Bubendorf, Switzerland) and isosorbide dinitrate (ISDN) from Schwarz Pharma (Watford, UK).

A schematic representation of the protocol is shown in **Figure 1**. Measurements began once a steady baseline was achieved during intracoronary infusion of 0.9% saline. Substance P was then infused for 2 minutes at 20pmol/min, a dose that elicits eNOS-mediated

endothelium-dependent vasodilation without inducing systemic effects (24). Following a washout period with normal saline, a 1mg ISDN bolus was administered to assess endothelium-independent vasodilatation (32). After a further saline washout, the Stroop color-word test was performed to elicit mental stress (15). This was followed by a recovery period after which SMTC (0.625 μ mol/min) was infused for 7 min. This dose of SMTC has previously been shown to provide selective inhibition of nNOS in the coronary circulation in a similar patient population (32). SMTC infusion was continued while the Stroop test was repeated. In 2 patients, a second Stroop test was performed during saline instead of SMTC infusion, in order to confirm that it evoked a reproducible increase in blood flow. Aortic pressure, heart rate and APV were recorded at baseline and after each phase of the protocol and coronary angiography was performed to quantify coronary diameter. The ECG was continuously monitored.

Statistical analyses. Subject characteristics are summarized as mean \pm SD; other results are presented as mean \pm SEM. Vasodilator/constrictor responses were analyzed both as absolute change in coronary flow and as percentage change from the immediately preceding baseline. Responses were compared by analysis of variance for repeated measures with adjustment for baseline values of coronary blood flow where appropriate (ANOVA / ANCOVA). Bonferroni correction for multiple comparisons was applied. All tests were 2-tailed and differences were considered significant at $p < 0.05$.

RESULTS

Baseline subject characteristics are shown in **Table 1**. The indications for coronary angiography included chest pain (7 subjects), dyspnea (2 subjects), non-sustained VT on Holter monitoring (1 subject) and pre-operative assessment in valvular disease (1 subject). Four subjects underwent functional testing for ischemia (either stress echocardiography, myocardial perfusion scintigraphy, or exercise treadmill testing), which was positive in 3 cases. Ten subjects had all three coronary arteries smooth and unobstructed; the 11th subject had minor irregularities (<10% stenosis) in all arteries. None of the subjects developed

adverse reactions, symptoms or ECG changes of ischemia during the infusions or mental stress.

Effect of mental stress and SMTC on coronary flow

The hemodynamic responses and changes in coronary blood flow and coronary conductance following mental stress are shown in **Table 2**. The changes in heart rate and blood pressure during mental stress were minimal, and similar with or without SMTC.

Mental stress increased coronary flow by $34 \pm 7.0\%$ ($p < 0.01$) (**Figure 2A**). SMTC reduced basal coronary flow by $20 \pm 4.7\%$ ($p = 0.01$), consistent with previous work (32, 33). It also significantly attenuated the vasodilator effect of mental stress, reducing the coronary flow response to a $26 \pm 7.0\%$ increase, compared to the preceding baseline (**Figure 2B**).

Substance P increased basal coronary flow by $24 \pm 8.0\%$ ($p = 0.01$). There was no significant correlation between the response to substance P and that to mental stress ($r^2 = 0.22$, $p = 0.83$). ISDN increased coronary flow in all subjects (mean increase of $119 \pm 40\%$) indicating an intact smooth muscle response to NO.

Effect of mental stress and SMTC on epicardial coronary diameter

Mental stress increased coronary artery diameter by $6.9 \pm 3.7\%$ ($p = 0.02$, **Figure 3**). SMTC reduced coronary artery diameter by $5.1 \pm 1.6\%$ ($p < 0.01$), and abolished the mental stress-induced increase to $0.5 \pm 2.8\%$ ($p = 0.98$ compared to preceding baseline). ISDN increased diameter by $3.9 \pm 2.0\%$ ($p = 0.01$) and substance P increased it by $3.1 \pm 1.6\%$ ($p = 0.07$). There was no significant correlation between the % change in coronary diameter and % change in coronary blood flow in response to acute mental stress ($r^2 = 0.19$, $p = 0.15$).

DISCUSSION

NO plays an important role in the regulation of vasomotor tone in the human coronary circulation, both during resting conditions and under situations of increased metabolic demand. At rest, it maintains a state of tonic vasodilation in resistance vessels (21) and conduit epicardial arteries (22). Following stimuli such as mental stress and cold pressors (3, 9, 13, 26), NO mediates dynamic changes in vascular tone that antagonize catecholamine-mediated vasoconstriction.

Until relatively recently, investigations of the role of NO in regulating coronary vascular tone in humans relied mainly on the use of the non-selective NOS inhibitor L-N^G-monomethyl arginine (L-NMMA) or of endothelium-dependent agonists such as acetylcholine, substance P and fluid shear stress (14, 21, 36-38). Based on such studies, eNOS expressed in the endothelium was assumed to be the main source of local NO that regulated vascular tone. However, experimental animal studies indicate that nNOS expressed in perivascular nerves or the vascular smooth muscle might also regulate local vessel tone (25). In line with this, nNOS is reported to be expressed in human coronary artery smooth muscle cells (18) and coronary perivascular nerves in rats and dogs (34, 44).

In first-in-man studies with the nNOS-selective inhibitor, SMTC, we previously demonstrated that basal blood flow in the human coronary circulation *in vivo* is under tonic regulation by local nNOS-derived NO (32). In these studies, we found that SMTC had no effect on endothelium-dependent vasodilation induced by substance P, which was however inhibited by L-NMMA. Similarly, local SMTC infusion into the forearm circulation of healthy human subjects reduced basal blood flow in an L-arginine-dependent manner but without affecting the vasodilator response to acetylcholine (33). Furthermore, we found that mental stress-induced increases in forearm blood flow in healthy men were significantly blunted by local intra-arterial infusion of SMTC, suggesting that local nNOS was involved in this setting. Previous work in animals has shown that nNOS-dependent effects on vessel tone vary significantly by vascular bed (10, 19, 23) and so it was important to establish whether nNOS is involved in mental stress-induced vasodilatation in the human coronary circulation.

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The novel finding of this study is that nNOS is responsible for mediating mental stress-induced vasodilation in the resistance vasculature of the human coronary circulation. The changes in coronary flow and conductance were not related to significant changes in systemic hemodynamics, suggesting that they represented a direct vasodilator action of nNOS-derived NO and not effects secondary to altered myocardial oxygen demand. This role of nNOS contrasts to pacing-induced increases in coronary blood flow, which we recently reported were mediated by eNOS-derived NO (35). The activation of eNOS during pacing is likely to involve increased endothelial shear stress whereas nNOS activation during mental stress may involve perivascular nitrergic nerves in the coronary vessel wall and their autonomic activation (2, 5).

The baseline response to mental stress in the current study was variable, ranging from a 1 to 77% increase in blood flow, similar to our previous observations in the forearm. Previous studies on mental stress-induced vasodilation in humans have reported average increases ranging from a 55% increase in blood flow (9) to a 10% increase in flow (43), which may be related to the differing characteristics of study participants. Specific factors that could have influenced the vascular response to stress in our study include vasoactive medications, body mass index and diabetes. Local characteristics of the vessel studied could also have influenced the response, although we selected vessels that were angiographically free of stenosis. The use of different techniques to elicit mental stress may also contribute to variation in responses. In the current study, SMTC reduced basal coronary flow as previously documented (32, 33), and substantially attenuated the mental stress-induced increase in blood flow. The degree of attenuation in stress-induced vasodilation is similar to that previously observed in the forearm (20), although it should be noted that subject characteristics were significantly different between these studies. The current study by necessity was undertaken in patients undergoing diagnostic coronary angiography and therefore included older patients with multiple risk factors for coronary disease and on oral medications.

Impaired mental stress-induced increases in flow have been reported in coronary artery disease (9, 31), metabolic syndrome (8), and non flow-limiting atherosclerosis (43).

215 Interestingly, we found little correlation between the response to substance P and the response
216 to mental stress, suggesting that endothelial eNOS-mediated vasodilator function may not be
217 the major determinant of the mental stress response. It is possible therefore that the variations
218 in the mental stress response could in part be explained by the presence of nNOS dysfunction
219 in these conditions (7, 30, 39). Potential underlying mechanisms include nNOS uncoupling,
220 which has been documented in conditions of oxidative stress such as atherosclerosis, and
221 increased levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA),
222 which has been observed in conditions such as diabetes and hypertension (29).

223 Numerous studies have been performed to explore the impact of stress on patients
224 with coronary artery disease, and a recent meta-analysis found that mental stress-induced
225 ischemia is associated with an approximately doubled risk of cardiac events (myocardial
226 infarction, revascularization and unstable angina) and total mortality (42). The finding of
227 reduced coronary blood flow during mental stress has also been shown to be a predictor of
228 daily ischemia in patients with coronary artery disease, independent of exercise-induced
229 ischemia (4).

230 Little is known about the long-term outcomes associated with impaired mental stress-
231 induced vasodilation in people who have been diagnosed with “normal coronary arteries”.
232 There are some important considerations to bear in mind. Firstly, it is now established that
233 coronary angiography can underestimate the degree of plaque burden compared to
234 postmortem histology (40). Secondly, a degree of “visual-functional” mismatch occurs on
235 coronary angiography, which can under or over- estimate a plaque’s functional severity in up
236 to 40% of cases (28). Thirdly, it is increasingly understood that the majority of acute coronary
237 syndromes arise from plaques that are not necessarily flow-limiting in size but share
238 vulnerable characteristics that ultimately lead to their rupture (6). Indeed, Yeung et al found
239 that mental stress triggers vasoconstriction at the site of both obstructive and non-obstructive
240 plaques in epicardial arteries, with an accompanying decrease in myocardial blood flow (43).
241 Additionally, Arrighi et al found that mental stress caused increased coronary resistance and
242 impaired myocardial blood flow in regions subtended by vessels without significant stenosis

(1). In the current study, we observed nNOS-dependent changes in epicardial coronary artery diameter as well as coronary blood flow in response to acute mental stress. These effects were not correlated with each other, suggesting that they may both reflect direct effects of nNOS, but we cannot exclude the possibility that the changes in epicardial diameter might be secondary to the changes in blood flow. Given the identification of nNOS as a mediator of stress-induced coronary flow in individuals with angiographically normal arteries, it would be of interest to examine its function in patients with established coronary disease.

In conclusion, this study identifies nNOS as the major NOS isoform responsible for mediating the vasodilator response to mental stress in the human coronary circulation. Due to the complex interplay that exists between mental stress and vasodilator function in the coronary circulation, dysfunctional blood flow responses to mental stress may contribute to the development of stress-induced myocardial ischemia, irrespective of the presence of angiographically significant coronary artery disease.

Study limitations

We estimated coronary blood flow from flow velocity rather than using thermodilution, but flow velocity has been widely used in studies of coronary vascular function. All the participants in our study either had risk factors for vascular endothelial dysfunction and/or were on vasoactive medications, so that the current results do not necessarily reflect the role of nNOS in completely healthy humans. To minimize potential confounding effects of these factors, we studied arteries that appeared angiographically smooth and unobstructed and we omitted medications on the day of the study. In addition, the impact of co-existing atherosclerosis risk factors in this study was not sufficient to impair endothelium-dependent vasodilation to substance P.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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FIGURE LEGENDS

Figure 1. Schematic representation of the infusion protocol. Average peak velocity, coronary angiography, BP, heart rate and ECG were recorded at each stage. ISDN, Isosorbide dinitrate; SMTC, S-methyl L-thiocitrulline.

Figure 2. Effect of S-methyl L-thiocitrulline (SMTC) on mental stress-induced increase in coronary blood flow (CBF). A, % change in CBF with the Stroop test during saline infusion (Stroop), SMTC, and the Stroop test during SMTC infusion. n=11, paired t-test between CBF during preceding baseline and intervention. * p<0.01 compared to preceding baseline. B, Absolute values for CBF under the different study conditions. n=11; *, significant interaction between groups by repeated measures ANOVA.

Figure 3. Changes in epicardial coronary artery diameter in response to interventions.

Percentage changes from the preceding baseline are shown. Sub P, substance P; ISDN,

nNOS and coronary flow during mental stress

451 isosorbide dinitrate; Stroop, mental stress test; SMTC, S-methyl l-thiocitrulline. n=11, paired
452 t test between diameter during preceding baseline and intervention. *, $p<0.05$ cf.
453 preceding baseline.
454

Table 1. Baseline characteristics of patients. Data are presented as mean \pm SD.

Characteristic		
n		11
Age (years)		58 \pm 14
Sex (M/F)		6/5
Hypertension, n		7
Diabetes mellitus, n		2
Hypercholesterolaemia, n		8
Smoker, n		0
BMI (kg/m²)		28.2 \pm 3.2
Medication: n (%)		
ACEI/ARB		4 (36)
β blockers or CCB		5 (45)
Statins		2 (18)
Study artery, n		
LAD		7
Cx		3
RCA		1

Table 2. Hemodynamic Responses During Mental Stress +/- SMTC

	Saline		SMTC	
	Baseline	Stress	Baseline	Stress
Heart rate (bpm)	59±3.7	60±3.9	55±3.0	62±4.9
Systolic BP (mmHg)	117±3.7	120±6.0	114±4.0	119±5.4
Mean BP (mmHg)	84±1.4	83±2.7	81±3.0	84±3.5
Diastolic BP (mmHg)	68±2.3	64±2.5	65±3.9	67±4.3
Coronary blood flow (ml/min)	38±3.9	52±5.4**	31±4.0	40±4.5**#
Coronary conductance (units)	0.49±0.04	0.61±0.08*	0.42±0.06	0.53±0.05*#

*p<0.05, **p<0.01 cf. preceding baseline; #, significant interaction between groups.

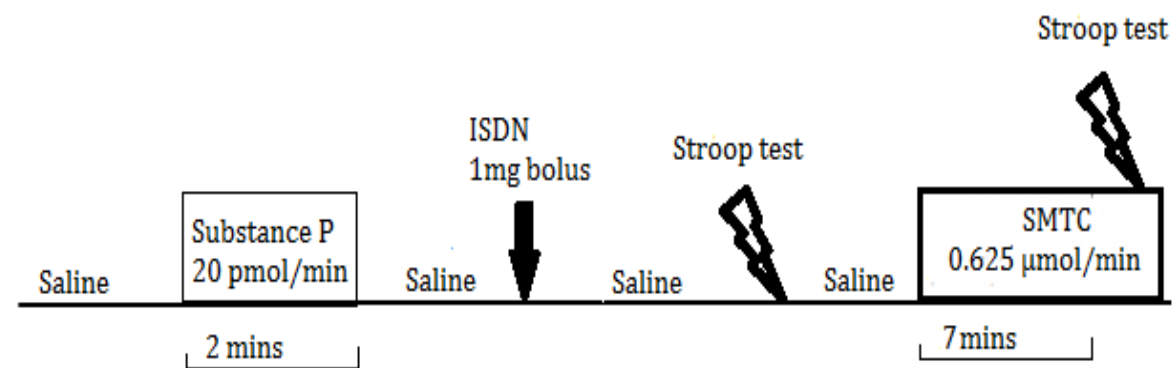


Fig. 1

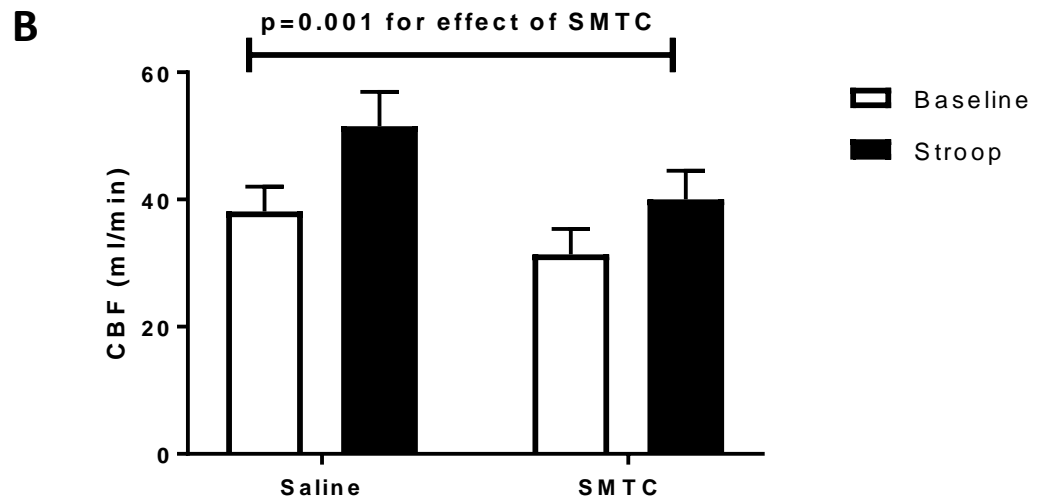
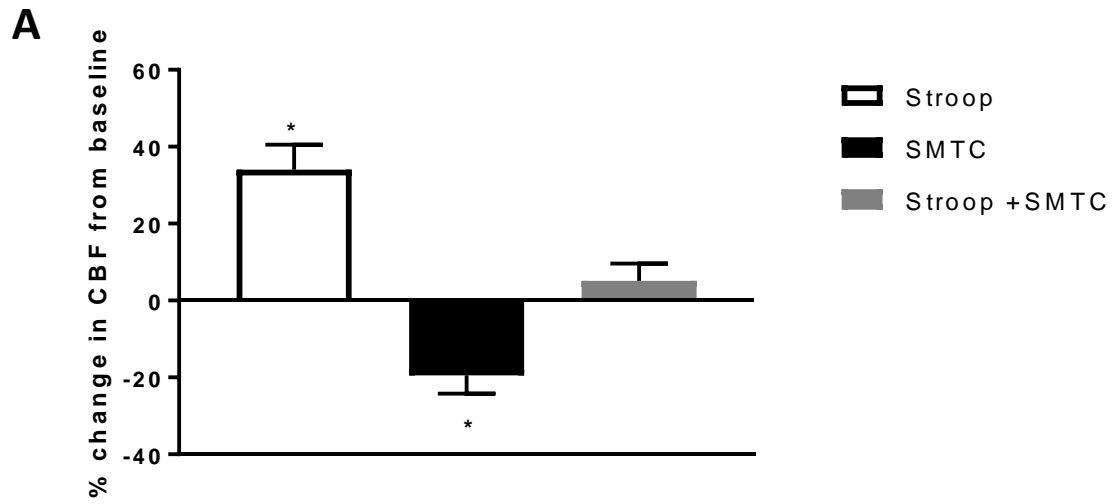


Fig. 2

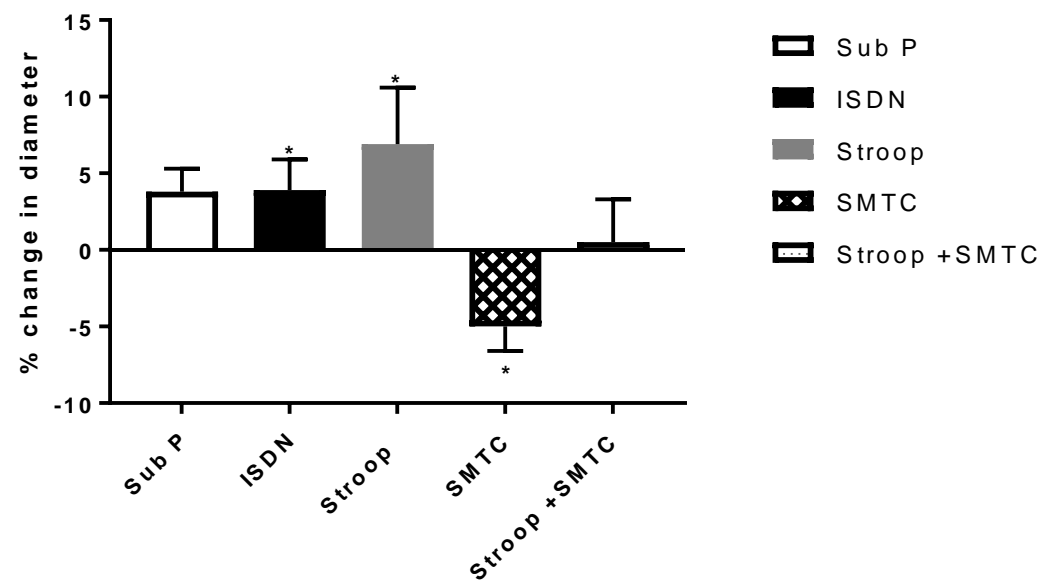


Fig. 3